Silver nanoparticle-mediated cellular responses in isolated primary Sertoli cells *in vitro*

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Keywords: Silver nanoparticles; Sertoli cell; DNA damage; oxidative stress; gene expression; endogenous antioxidants enzymes

Highlights

• AgNPs entered Sertoli cells and caused severe testicular oxidative damage and/or apoptosis.

• Exposure of the Sertoli cells to AgNPs produced significant increases in superoxide anions.

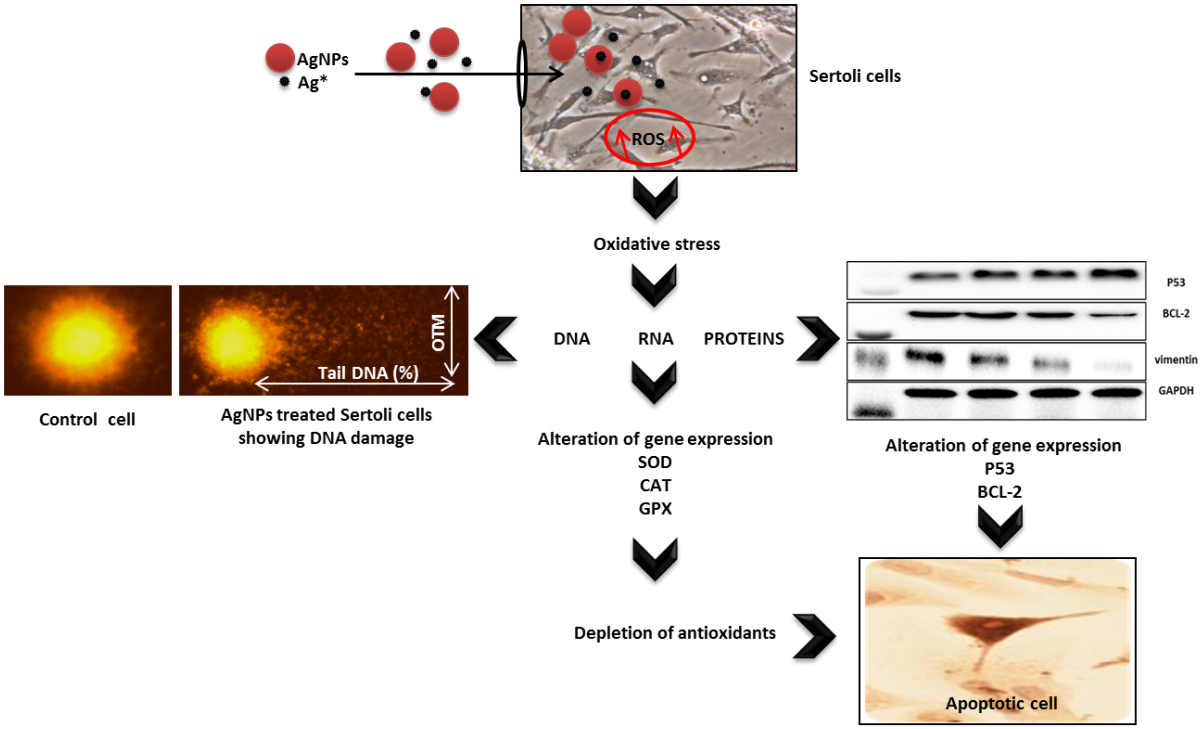
• Exposure to AgNPs activated p53, repressed bcl-2 and reduced endogenous antioxidant enzymes.

• The male reproductive system can be disrupted by exposure of somatic cells to AgNPs

Abstract

The present study explored the mechanism of cytotoxic and genotoxic effects of AgNPs on a primary culture of mouse Sertoli cells in vitro. To understand the possible molecular mechanisms of testicular lesions following exposure to AgNPs, isolated Sertoli cells were exposed to 5, 10, or 15 µg/ml. DNA damage in the Comet assay and apoptosis in the TUNEL assay were evaluated. The mRNA expression of p53 and bcl-2 genes and their proteins involved in apoptosis was also investigated. The antioxidant status of treated Sertoli cells was determined by measuring catalase (CAT), glutathione peroxidase (GPX-1) and superoxide dismutase (SOD-1) using qPCR. The superoxide anions were detected using the nitroblue tetrazolium (NBT) reduction assay. Results indicated that AgNP exposure causes increased oxidative stress levels. The activation of p53, repression of bcl-2 and reduction of endogenous antioxidant enzymes were also involved in these mechanistic pathways, leading to reduced cell numbers and cell detachment.

Graphical abstract



Introduction

Nanomaterials are becoming widely used and are increasingly important in biological, therapeutic, clinical medicine, and antimicrobial agents. Silver nanoparticles (AgNPs) are commonly used in many fields such as health care, pharmaceutical products, food industries, due to their unique physicochemical properties such as chemical, magnetic, optical and electrical biological (Gurunathan et al., 2015). Many studies have investigated the effects of AgNPs on vital developmental parameters of the reproductive system. In these studies, it has been confirmed that defined size and concentrations of AgNPs can cause male reproductive toxicity in mammals (Ong et al., 2016; Sleiman et al., 2013). AgNPs impaired the proliferation of mouse spermatogonial stem cells by interacting with Fyn kinase downstream of Ret (GDNF/Fyn) *in vitro* (Braydich-Stolle et al., 2010). In the rat model, AgNPs also adversely affected spermatogenic cells (Miresmaeili et al., 2013). In addition, an *in vivo* study showed that daily exposure to AgNPs during prepubertal development caused sperm abnormalities and decreased reproductive parameters in the adult male rat (Mathias et al., 2015). Mice which had received intravenous injections of AgNPs also showed effects in spermatocyte development (Han et al., 2016). Another, study has shown that male somatic Leydig (TM3) and Sertoli (TM4) cells were significantly affected after exposure to AgNPs (Zhang et al., 2015a).

The interplay between Sertoli and spermatogenic cells is crucially significant for successful development of germ cells into spermatozoa, because Sertoli cells form sites of attachment to spermatogenic cells. Spermatogenic cells which are lose in the seminiferous epithelium have been found frequently associated with Sertoli cell dysfunction (Gray and Beamand, 1984). Sertoli cells are responsible for the

orchestration and regulation of spermatogenic cells and provide nutrition (Aumuller et al., 1988).

In order to characterize the effects of AgNPs on isolated Sertoli cells, the present study was undertaken to examine the potential toxicological effects of Ag-NPs on isolated mouse Sertoli cells, a critical cell type of the male reproductive tract. In this study, DNA damage and apoptosis were evaluated; examination of mRNA levels of p53, bcl-2 and SOD, CAT and GPX gene expressions were quantified. Also superoxide anions production of the cultured Sertoli cells was assessed.

Materials and Methods

Chemicals

Silver nanospheres (CAS No. 795933) were purchased from the Sigma-Aldrich Company Ltd. (Sigma Chemical Ltd., Gillingham, UK). All other chemicals used in the different tests were from Sigma-Aldrich Company Ltd. (Sigma Chemical Ltd., Gillingham, UK).

Animals

Male adult National Medical Research Institute (NMRI) mice (aged 6-8 weeks old) were used in this study. Animals were obtained from the Institute of Cancer Therapeutics Laboratories, University of Bradford, UK where they were maintained under the standard conditions. All animal care procedures were carried out according to the National Research Council's Guide for the Care and Use of Laboratory Animals.

Isolation, identification, and culture of mouse Sertoli cells

Four male (NMRI) mice were sacrificed by cervical dislocation under CO_2 anaesthesia, the testes were immediately excised in aseptic conditions and transferred to cold calcium- and magnesium-free Hanks balanced salt solution (HBSS) containing 10000 units/ml penicillin, 10 mg/ml streptomycin and 25 µg/ml amphotericin B (pH 7.4) immediately after removal. Decapsulated tissue was washed twice in a 50 ml conical tube in 30 ml of ice-cold HBSS. Minced tissue was suspended in HBSS and shaken vigorously for 1 min to disperse tubules. The tissue was left to settle for 5 min on ice, and the supernatant was discarded. This procedure was repeated twice to mechanically remove red blood cells and free Leydig cells. The resulting pellet was digested (I) in 25 ml of HBSS containing collagenase type I 0.5 mg/ml collagenase and 0.04 mg/ml Dnase (both from Sigma, Poole, UK) and continuously shaken in a shaking water bath at 32°C for 20–25 min. The formed aggregates were removed, washed in HBSS and discarded. The washing HBSS was added to the cellular suspension resulting from the digestion and further digested (II) with the same enzyme for 20 min at 32°C. The new aggregate formed was discarded and 0.1ml fetal bovine serum (FBS) was added to the cellular suspension, which was left to rest at 4°C for 5min. The suspension was then centrifuged at 100 g for 5min. The pellet was gently suspended in 5ml HBSS. This procedure was repeated twice and the resulting pellet was suspended in 5ml HBSS. This suspension was passed through a glass pasteur pipette in order to loosen germ cells from the clusters, and then pelleted at 200 g for 5min. This procedure was repeated twice. The resulting pellet was suspended in Sertoli culture medium DMEM/F12 supplemented with 10% fetal bovine serum (FBS) containing 50U/ml

penicillin and 50 µg/ml streptomycin sulfate, 0.5 µg/ml fungizone and forced through a 19G needle, in order to disaggregate large Sertoli cell clusters.

For differential plating, Sertoli cells and germ cells were plated on 25cm^2 culture flasks and incubated at 37°C in an atmosphere of 5% CO_2: 95% O2. The day of plating was considered day 0 of culture. The cultures were left undisturbed until day two. Sertoli cells attached to the culture plates, whereas male germ cells remained in suspension and were removed. Cell viability of Sertoli cells was determined with trypan blue exclusion assay and the CC8 kit.

Treatment

The freshly isolated Sertoli cells were plated at a density of 2 × 10^6 cells/ml in DMEM/F12 supplemented with 10% FBS in a humidified incubator at 37°C in an atmosphere of 5% CO_2: 95% O2 for 24 h. The Sertoli cell-enriched cultures obtained were reseeded onto chamber slides. The samples were cultured for a further 5 days. The cells were treated with different concentrations of AgNPs at 0, 5, 10 and 15µg/ml. The treated and untreated Sertoli cells were used in the Comet assay, TUNEL assay, nitroblue tetrazolium (NBT) assay, qPCR and Western blot. Some of the untreated cells were processed for vimentin immunohistochemical analysis.

Immunohistochemistry assay

The identity and purity of Sertoli cells used in the study was confirmed by immunohistochemistry using the antibody against vimentin. The cells were grown in 8 well chamber slides with DMEM/F12 containing 10% FBS, 100 Unit/ml penicillin, and 100 mg/ml streptomycin for 24 h to allow the cells to attach. Slides were fixed with 4% formaldehyde for 20 min and washed twice, each for 5 min, in PBS, A 1 h

block in Phosphate-buffered saline (PBS) containing 0.1% BSA, 0.05% Triton X-100, and 1% goat serum was performed, and incubated at 4°C overnight with primary antibody (anti- vimentin rabbit monoclonal (1:200; Abcam, Cambridge, UK). The sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. Signals were developed with 3,3′-diaminobenzine (DAB) for 10 min and counterstained with haematoxylin and slides were applied using Histomount mounting medium (Fisher Scientific, Fair Lawn, NJ). For a negative control, the incubation step with the primary antibody was absent.

Cytotoxicity assays

Cell viability was determined using a modified cell counting kit-8 (CCK-8) Cytotoxicity Assay (Sigma-Aldrich, UK). Isolated Sertoli cells were seeded onto a 96-well plate and incubated overnight in 95% humidity, 5% CO_2 at 37 °C. After 24 h, the medium was replaced with a fresh one containing AgNPs in different concentrations (0, 5, 10 and 15 µg/mL) for 24 h. CCK-8 solution (10 µl) was added to each well, followed by incubation for 4 h at 37°C. The absorbance at 450 nm was determined by a using a Microplate reader MRX II (Dynex Technologies, Chantilly, USA).

Evaluation of superoxide anion levels

The production of superoxide anion by AgNP was quantified through the NBT-reduction assay. Typically, isolated Sertoli cells were plated onto 24 well cell culture plates in complete DMEM/F-12 medium at a density of 5×10^5 cells per well and incubated at 37 °C and 5% CO_2. The cells were washed twice with PBS and then cells were treated with AgNP at concentrations (0, 5, 10 and 15 µg/ml) for 1 h. The medium was replaced with phenol red-free media (M199) (Sigma-Aldrich, UK), containing 1 mg/ml NBT with and without (0, 5, 10 and15 µg/ml) AgNPs; the mixture

was incubated at 37°C under 5% CO_2 for 90 min. Sertoli cells were lysed with lysis solution 90% DMSO, 0.1% SDS and 0.01 M of NaOH. The production of intracellular superoxide anion was measured spectrophotometrically at a wavelength of 750 nm using microplate reader MRX II (Dynex Technologies, Chantilly, USA).

Evaluation of DNA damage in the Comet assay

The Comet assay was performed to quantitate DNA damage in Sertoli cells. The alkaline comet assay was used according to the methods by Anderson et al. (1997) with certain modifications: Briefly, after treatment isolated Sertoli cell debris was removed and cells remaining in the plates from each treatment were harvested by centrifugation and then used for the examination of DNA damage according to protocol as described previously (Habas et al., 2016).

Detection of apoptosis by the TUNEL assay

The apoptotic cells were evaluated on isolated Sertoli cells by the TUNEL assay using a commercial apoptosis detection kit (Terminal Deoxynucleotidyl Transferase Detection Kit; Promega, UK, Ltd). TUNEL analysis was performed to quantify cell apoptosis on isolated Sertoli cells a commercial apoptosis detection kit (Terminal Deoxynucleotidyl Transferase Detection Kit; Promega, UK, Ltd). Cells were cultured with 0, 5, 10 and 15 µg/mL AgNPs for 24 h, and then washed in PBS, fixed in 4% paraformaldehyde for 15 minutes, and incubated in a TUNEL reaction mixture for 1 hours at 37°C, according to the procedure as described previously (Habas et al., 2014).

RNA extraction and real-time PCR

Total RNA was extracted from isolated primary Sertoli cells with and without different concentrations of AgNPs (0, 5, 10 and15 µg/mL) for 24 h using TRIzol® following the manufacturer's (Invitrogen) instructions. Reverse transcription was performed using an ImProm-II™ Reverse Transcription System reaction according to the procedure as described previously (Habas et al., 2017).The expression of each gene was detected by real-time quantitative PCR, with the housekeeping gene β-actin used as an internal control. The reactions were performed in MicroAmp optical 96-well plate using a Step OnePlus™ real-time PCR instrument (Applied Biosystems) using SYBR® Green PCR Master Mix (Applied Biosystems). The data obtained from each reaction was analyzed by StepOne™ Software v 2.2.2. The transcript levels of each specific gene were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Western blot analysis

The cells were treated as above and lysed in lysis buffer. Protein concentrations were determined using a commercial kit (Bradford Protein Assay, Biorad, UK) according to the manufacturer's specifications. Proteins were separated on 12% polyacrylamide gels and transferred to polyvinylidenefluoride membranes. These membranes were incubated for 1 h at room temperature in Tris-buffered saline containing 0.5% non-fat milk powder, and 0.1 % Tween-20. An antibody against vimentin (Abcam, Cambridge, UK), an antibody against p53 (Abcam, Cambridge, UK), and an antibody against bcl-2 (Abcam, Cambridge, UK) were used. An antibody against GAPDH (diluted 1:1,000; Abcam, Cambridge, UK) was used as the positive control. After incubation with the primary antibody overnight at 4 °C, the membranes

were washed three times in TBS. After incubation with the secondary horseradish peroxidase-conjugated antibodies (1:1,000; Abcam, Cambridge, UK) for 1 h at room temperature, detection was performed using an enhanced chemiluminescence assay kit (GE Healthcare, UK). Digital images were captured and density measurements were made using commercial software (Quanity One, Biorad).

Statistical analysis

Data are expressed as mean ± SEM of at least three independent experiments. Differences between the means were tested by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons using the Graph-Pad Prism software. The differences were considered significant at $P<0.05$.

Results

Isolation, identification, and culture of primary Sertoli cells

Sertoli cells from adult mouse testes were isolated by a two step enzymatic digestion and followed by differential plating. Cell viability was up to 96% as assayed by trypan blue exclusion. After 24 hours of culture, Sertoli cells attached to the dish and assumed a large columnar or irregular appearance with an elongated cellular body. The identity and purity of cells used in the experiments was confirmed by an immunohistochemistry assay, because the isolated cells are contaminated with other testicular cells, mainly myoid cells. Thus, our results confirmed that the isolated cells were indeed Sertoli cells. The purity of isolated Sertoli cells was more than 95 % as assayed by immunocytochemical staining with vimentin antibody (Figure 1).

Effects of AgNPs on isolated primary Sertoli cells viability

To assess the cytotoxicity of AgNPs on Sertoli cells under nonstarved conditions cell viability was investigated using a using CCK-8 kit. Sertoli cells were either treated with different concentrations of AgNPs (0, 5, 10 and 15 µg/ml) or left untreated and considered as control. AgNPs at the concentration 15 µg/mL there was a significant effect on cell viability of primary Sertoli cells (*$p \leq 0.05$) at 24 h (Figure 2).

Detection of intracellular superoxide anion production on primary Sertoli cells.

The generation of superoxide anion was detected by the NBT assay. Primary Sertoli cells treated with AgNPs for 1 h showed increased production of superoxide anion which were significantly increased compared to non-treated cells (**$p < 0.01$) and (***$p < 0.001$) (Figure 3).

Effects of AgNPs on DNA damage of primary Sertoli cells

The responses of isolated Sertoli cells to AgNPs for the Comet assay parameters Olive tail moment (OTM) and percent DNA in tail (% tail DNA) are shown in Figure 4 A and B, A significant increase was seen in OTM and % tail DNA in the Sertoli cells from 3.02 (OTM) and 12.73 % (% tail DNA) compared to the untreated control groups to 0.68 (OTM) 4.97 % (% tail DNA), respectively, when cells were treated with 10 ug/ml AgNPs. Further increases to 4.77 in (OTM) and 15.44 % in (% tail DNA) were observed when cells were treated with 15 ug/ml AgNPs respectively.

Effects of AgNPs on primary Sertoli cells apoptosis detected by TUNEL assay

Isolated primary Sertoli cells were exposed to AgNPs with different concentrations for 24 h. Later stage of cell apoptosis was observed and expressed as mean percentage per group ± SEM of apoptotic cells. Figure 5 showed TUNEL-positive

cells in the primary Sertoli cells had undergone significant levels of apoptosis compared with the controls (*$p \leq 0.05$). After treatment with AgNPs (0, 5, 10 and 15 µg/mL), the high concertation of AgNPs led to more TUNEL-positive cells (Figure 5 B, C, and 5D) compared to the control (Figure 5A) and lower concentrations of AgNPs (Figure 5B).

Effects of AgNPs on mRNA expression and protein levels

We further evaluated gene expression and protein in primary Sertoli cells with or without AgNPs exposure. The expression level of the *B*-actin gene was used gene as a reference; changes in the expression levels of the SOD, CAT and GPX oxidative stress and/or apoptosis-related genes p53 and bcl-2 were evaluated and compared following exposure to AgNPs for 24 h (Figures 6 and 7). qPCR revealed that mRNA expression of SOD, CAT, GPX and bcl-2 decreased gradually, whereas the expression levels of p53 increased significantly in primary Sertoli cells with increasing AgNPs concentrations (*$p < 0.05$, **$p < 0.01$ and ***$p<0.001$) respectively Figure 6A. Western blot analysis was used to evaluate p53 and blc-2 proteins expression levels in Sertoli cells following AgNPs treatment. Our data showed that p53 protein expression levels gradually increased with increasing concentrations of AgNPs, whereas the expression levels of bcl-2 decreased significantly in primary Sertoli cells with increasing AgNPs concentrations (*$p < 0.05$ and **$p < 0.01$) respectively (Figure 6 B and 6D). AgNPs-induced tight junction-related molecular expression was also examined following exposure to various concentrations of AgNPs for 24 h (Figure 8A). Western blot analysis showed that AgNPs caused significant reductions in vimentin protein expression in mouse isolated primary Sertoli cells (**$p < 0.01$ and ***$p<0.001$), respectively (Figure 8B and 8C).

Discussion

In spite of the many benefits of nanotechnology, various studies recently state that certain nanoparticles (NPs) may cause adverse effects on the reproductive system functions because of their unique properties (Zhang et al., 2015b; Zhang et al., 2015c). A study has been reported that exposure to NPs may cause alteration in embryogenesis, anomalies in the fetal reproductive system and a compromised fertility (Takeda et al., 2009). In the male reproductive system, Sertoli cells are mesoepithelial somatic cells that coordinate and structurally support the maturing germ cells. Damage to these cells eventually affect sperm production. To understand the mechanisms of oxidative stress and apoptosis, the expression of oxidative stress and apoptosis-related genes in the Sertoli cells was assessed. It has been reported that oxidative stress can affect testicular functions important for spermatogenesis (Zhang et al., 2013). ROS including superoxide anion are highly reactive intermediates best known for their ability to induce cellular damage (Bronsart et al., 2016). The quantification of superoxide anion production has been commonly used to assess adverse environmental conditions and pathogenesis of various chronic diseases including cancer and inflammation (Finkel and Holbrook, 2000; Waris and Ahsan, 2006). Our results show that cells treated with AgNPs caused significant increases in the production of superoxide anions compared to non-treated cells (Figure 3). Production of cellular ROS is typically a factor contributing to cell death and DNA damage (Lee et al., 2014). Exposure to AgNPs also significantly increased levels of DNA strand breaks in both OTM and % tail DNA with the increase of AgNPs concentration (Figure 4 A and B). Excessive production of ROS can directly trigger both intrinsic and extrinsic apoptotic pathways. The activation of p53 plays a key and complex role in cellular responses to oxidative stresses (Sablina et al., 2005). Our

results showed that isolated primary Sertoli cells treated with AgNPs resulted in increases in p53 (Figure 6), suggesting that the p53 pathway mediates AgNP induced cellular cytotoxicity. Similar findings were reported with the anti-apoptotic bcl-2 which was significantly decreased with increasing concentrations of AgNPs (Figure 6). Overexpression of bcl-2 prevents cells from undergoing apoptosis in response to a variety of stimuli. Cytosolic cytochrome c is necessary for initiation of the apoptotic programme, suggesting a possible connection between bcl-2 and cytochrome c, which is normally located in the mitochondrial intermembrane space. Our results are concordant with earlier reports; AgNPs induced apoptosis via activation of p53 and repression of bcl2, ultrastructural changes in the TM3 and the TM4 cell lines (Zhang et al., 2015b). Isolated primary Sertoli cells exposed to titanium dioxide nanoparticles (TiO_2 NPs) results in apoptosis in mice (Ritz et al., 2011).

AgNPs-induced oxidative damage in the primary Sertoli cells responded to modulation of antioxidant capacity. AgNPs generated elevated intracellular ROS and down-regulation of antioxidant enzymes such as GPX and SOD, resulting in the formation of DNA adducts (AshaRani et al., 2009; Carlson et al., 2008). This enzymatic defence includes SOD, CAT and GPX which are important antioxidant enzymes that are required for maintaining physiological levels of $O_2^{\cdot-}$ to H_2O_2 and all are expressed in the testis (Maiorino et al., 2003; Zini and Schlegel, 1997). The SODs are a group of enzymes that rapidly catalase conversion of $O_2^{\cdot-}$ to H_2O_2 and O_2, GPX and CAT are the major enzymes that regulate the cellular peroxidase level and reduce H_2O_2 to H_2O and O_2. In the present study, data showed that exposure to AgNPs significantly decreased in the levels of mRNA SOD, CAT and GPX, resulting

in a higher level of ROS production and DNA damage in Sertoli cells following exposure to AgNPs (Figure 7).

A study has shown that administration of NPs to mice results in their accumulation in the testis. Because of their small size, NPs can cross many barriers. This indicates that they easily pass through the blood–testis barriers and some of them have toxic action on male germ cells (Borm and Kreyling, 2004; Chen et al., 2003; Tsukue et al., 2004). Sertoli cells provide a favourable environment for the growth and differentiation of germ cells. The last stage of germ cells, namely the spermatozoa is highly rich in polyunsaturated fatty acids and very vulnerable to attack by ROS (Murugesan et al., 2005; Sikka, 2001). The protection against lipid peroxidation is due mainly to the activity of GPX, and it plays a critical role in maintenance of sperm viability and motility (Hu et al., 2010). Together, these findings underscore the importance of antioxidant enzymes in preventing DNA damage and/ or the apoptosis processes.

Vimentin is very important Sertoli cell cytoskeleton component and plays a critical role in positioning the Sertoli cell nucleus and anchoring spermatogenic cells to the seminiferous epithelium (Vogl et al., 2008). Vimentin genes encode tight junction proteins and play a critical role for the formation of the blood–testes barrier (Zhang et al., 2015b). Because of the essential role of vimentin in the control of Sertoli cells, we next examined the expression of tight junction genes and their proteins in AgNPs-treated primary Sertoli cells. Our data showed that vimentin expression was decreased in isolated primary Sertoli cells after treatment with AgNPs, implying that they may influence the spermatogenesis development. It has been reported that vimentin expression was downregulated in TM4 cells after the exposure to AgNPs (Zhang et al., 2015b). Dysfunction of these cells could affect spermatogenesis and

lead to the reduction and attachment of spermatogenic cells. Our results suggest that AgNPs inhibit endogenous antioxidant enzymes of mouse primary Sertoli cells *in vitro*. The decrease of anti-oxidative stress enzymes could be associated with induced DNA damage and/or apoptosis of Sertoli cells and may impair the capacity of these cells against oxidative damage by reducing their own active oxygen production and lipid peroxidation, which may adversely affect spermatogenesis and male reproduction.

Conclusions

In this study, we demonstrated that AgNPs entered Sertoli cells and caused severe testicular oxidative damage and/or apoptosis, accompanied by excessive production of the superoxide anion, proteins and DNA as well as a significant reduction in the endogenous antioxidant enzyme. Furthermore, exposure to AgNPs resulted in the up-regulation of p53 and caused down-regulation of SOD, CAT, GPX, and bcl-2 expression in isolated primary Sertoli cells from mouse testis. Our findings have provided biological insights into cell signalling pathways causes the toxicity of AgNPs in Sertoli cells, also highlighting the importance of risk assessment of AgNPs when used in consumer or biomedical applications.

Acknowledgement

The Sponsorship of the Libyan Government of a PhD studentship to Khaled Habas is gratefully acknowledged. The Sponsor played no part in the conduct of the work or the writing of the manuscript.

Conflict of interest

The authors have no conflicts of interest with regard to the funding of this research.

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Figure legend

Figure 1. Identification of the isolated Sertoli cells. A: isolated Sertoli cells cultured, B: unstained isolated Sertoli cells (negative control) C: The identity and purity of cultured Sertoli cells was confirmed by immunostaining with an antibody against Sertoli cell-specific vimentin protein.

Figure 2. Evaluation of cytotoxicity of different concentrations of AgNPs for 24 h on isolated primary Sertoli cells using the cytotoxicity assay (CCK-8). The results are expressed as means ± SE obtained from three independent experiments. *$p < 0.05$ when compared with the respective control group.

Figure 3. Effect of AgNPs on superoxide anion production in isolated primary Sertoli cells using the NBT assay. Cells were grown in complete medium/F12 and treated with and without 0, 5, 10 and 15 µg/mL for 1 h. Non-treated cells were considered as negative control. The results are expressed as means ± SE obtained from three independent experiments. **$p < 0.01$ and ***$p < 0.001$ versus non-treated cells.

Figure 4. A) DNA damage measured as mean OTM before and after treatment with AgNP in isolated primary Sertoli cells in the Comet assay. Data represent the means ± SE obtained from three independent experiments.**$P<0.01$ when compared with the respective control group. B) DNA damage measured as mean % tail DNA before and after treatment with AgNP in isolated primary Sertoli cells in the Comet assay. Data represent the means ± SE obtained from three independent experiments.**$P<0.01$ when compared with the respective control group.

Figure 5. TUNEL assay showed apoptotic cells in the isolated primary Sertoli cells with different concentrations of AgNP treatment. A-D: TUNEL-positive cells in the isolated primary Sertoli cells without AgNP treatment (A), or treated with AgNP at 5 µg/mL (B), 10 µg/mL (C), or with 15 µg/mL (D). E: Apoptotic quantitation assay showed that the mean percentages ± SEM of apoptotic cells at AgNP concentrations of 0, 5, 10 and15 µg/mL for 24 h, **$P < 0.01$ when compared with the respective control group. Arrows indicate representative TUNEL-positive (apoptotic) cells. Viewing magnification ×400.

Figure 6. (A): Alterations in the mRNA expression of apoptosis-related genes p53 and bcl-2 in isolated primary Sertoli cells caused by treatment with AgNPs for 24 h. (B): shows a representative blot indicating a protein band demonstrating p53 and bcl-2. (C): data (mean ± SE) are shown as a bar graph of densitometry data from three different experimentals (n=3). *$p < 0.05$, **$p<0.01$ and ***$p<0.001$ compared with the control group.

Figure 7. Alterations in the mRNA expression of antioxidative enzymes-related genes SOD, CAT and GPX in isolated primary Sertoli cells caused by treatment with AgNPs for 24 h. *$p < 0.05$ and **$p < 0.01$. Values represent mean ± SE (n = 3).

Figure 8. Effect of AgNPs on vimentin expression in isolated primary Sertoli cells. Cells were incubated with or without AgNPS for 24 h. (A): shows a representative blot indicating a protein band demonstrating vimentin. (B): data (mean ± SE) are shown as a bar graph of densitometry data from three different experimentals (n=3). **$p<0.01$ and ***$p<0.001$ compared with control group.

Figure 1

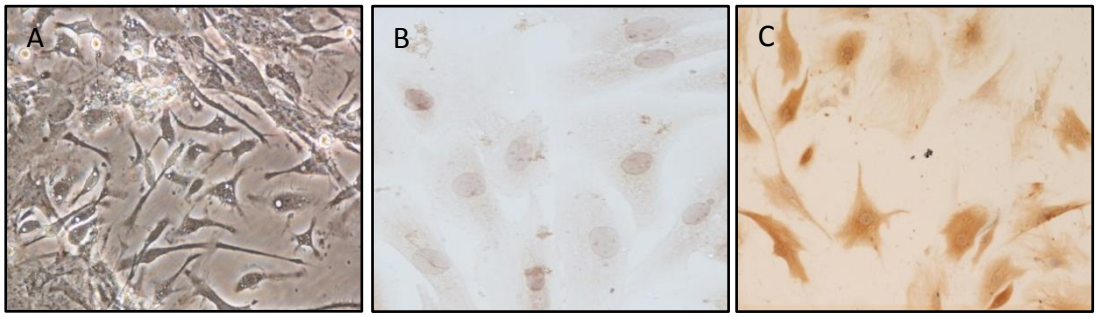


Figure 2

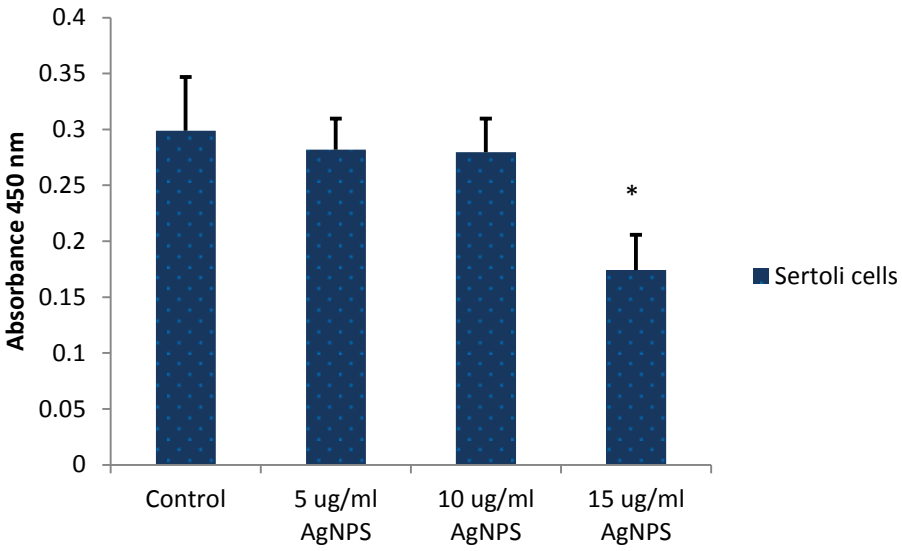

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance 450 nm
*
Control
5 ug/ml AgNPS
10 ug/ml AgNPS
15 ug/ml AgNPS
Sertoli cells

Figure 3

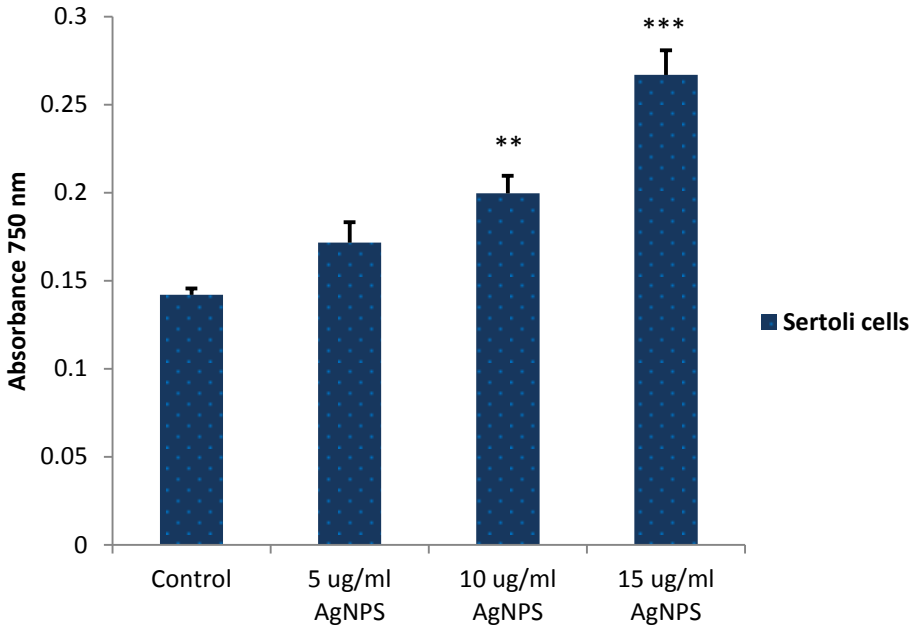

0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance 750 nm
**
***
Control
5 ug/ml AgNPS
10 ug/ml AgNPS
15 ug/ml AgNPS
Sertoli cells

Figure 4

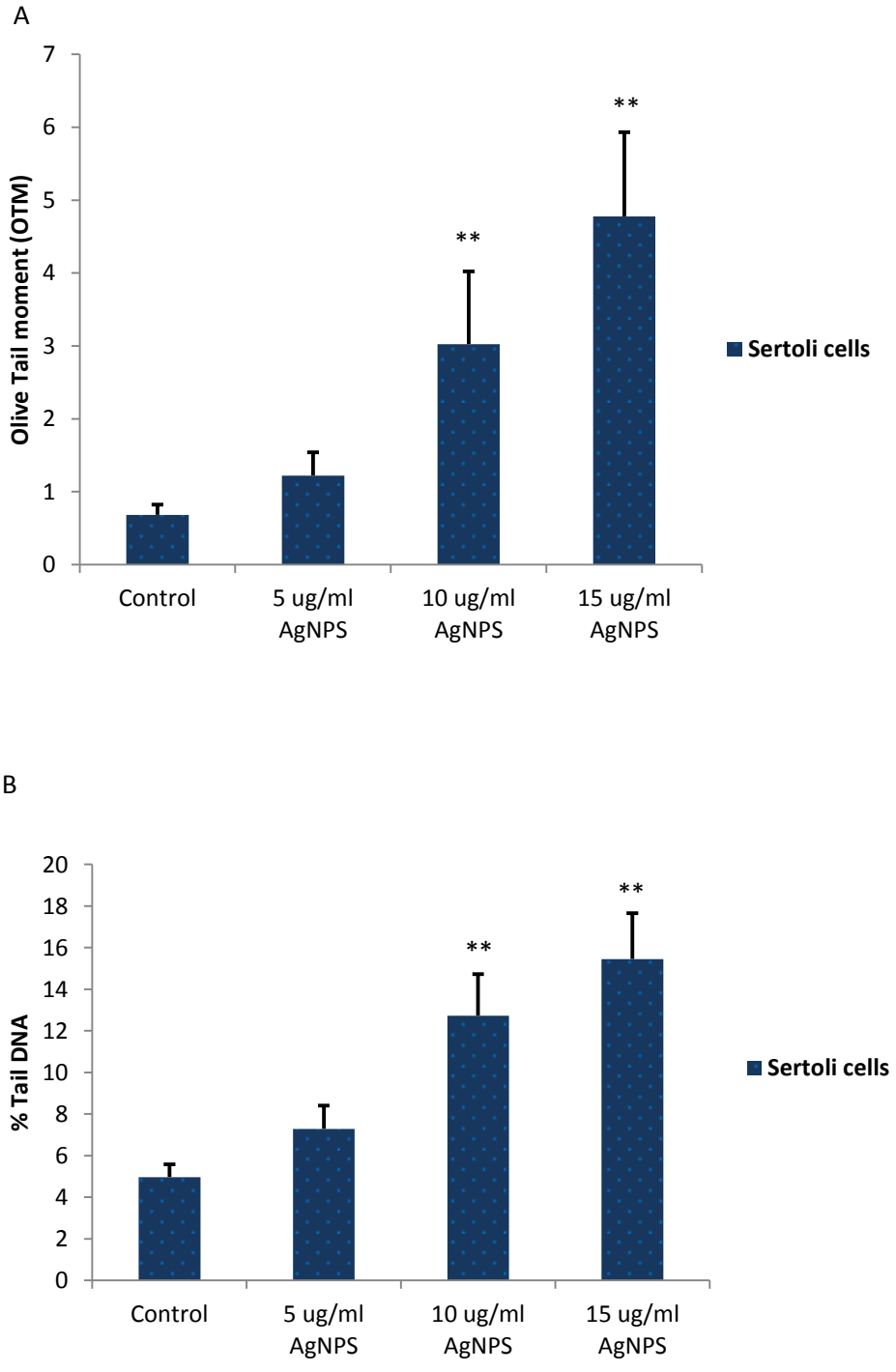
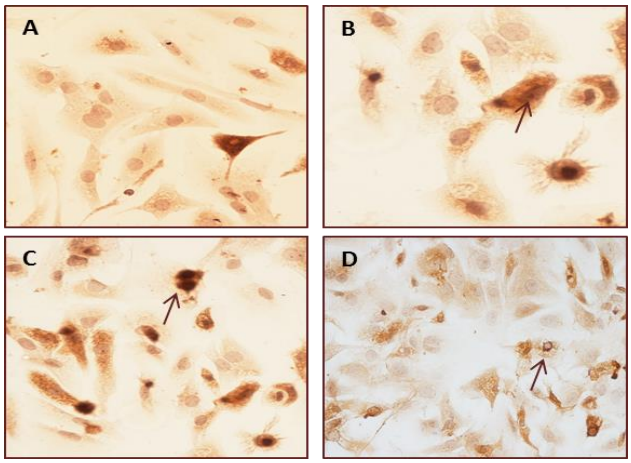

A
Olive Tail moment (OTM)
0
1
2
3
4
5
6
7
**
**
Control
5 ug/ml AgNPS
10 ug/ml AgNPS
15 ug/ml AgNPS
Sertoli cells
B
% Tail DNA
0
2
4
6
8
10
12
14
16
18
20
**
**
Control
5 ug/ml AgNPS
10 ug/ml AgNPS
15 ug/ml AgNPS
Sertoli cells

Figure 5


A
B
C
D

E

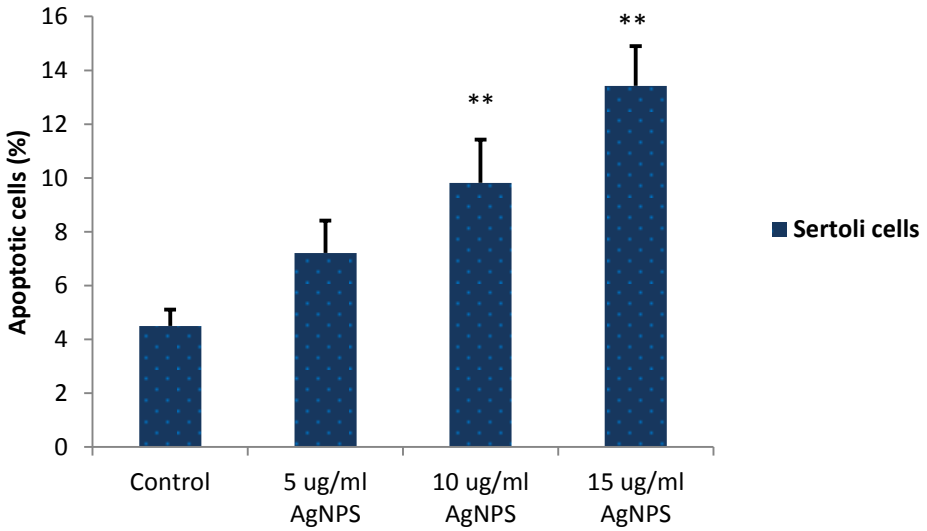

Apoptotic cells (%)
16
14
12
10
8
6
4
2
0
**
**
Control
5 ug/ml AgNPS
10 ug/ml AgNPS
15 ug/ml AgNPS
Sertoli cells

Figure 6

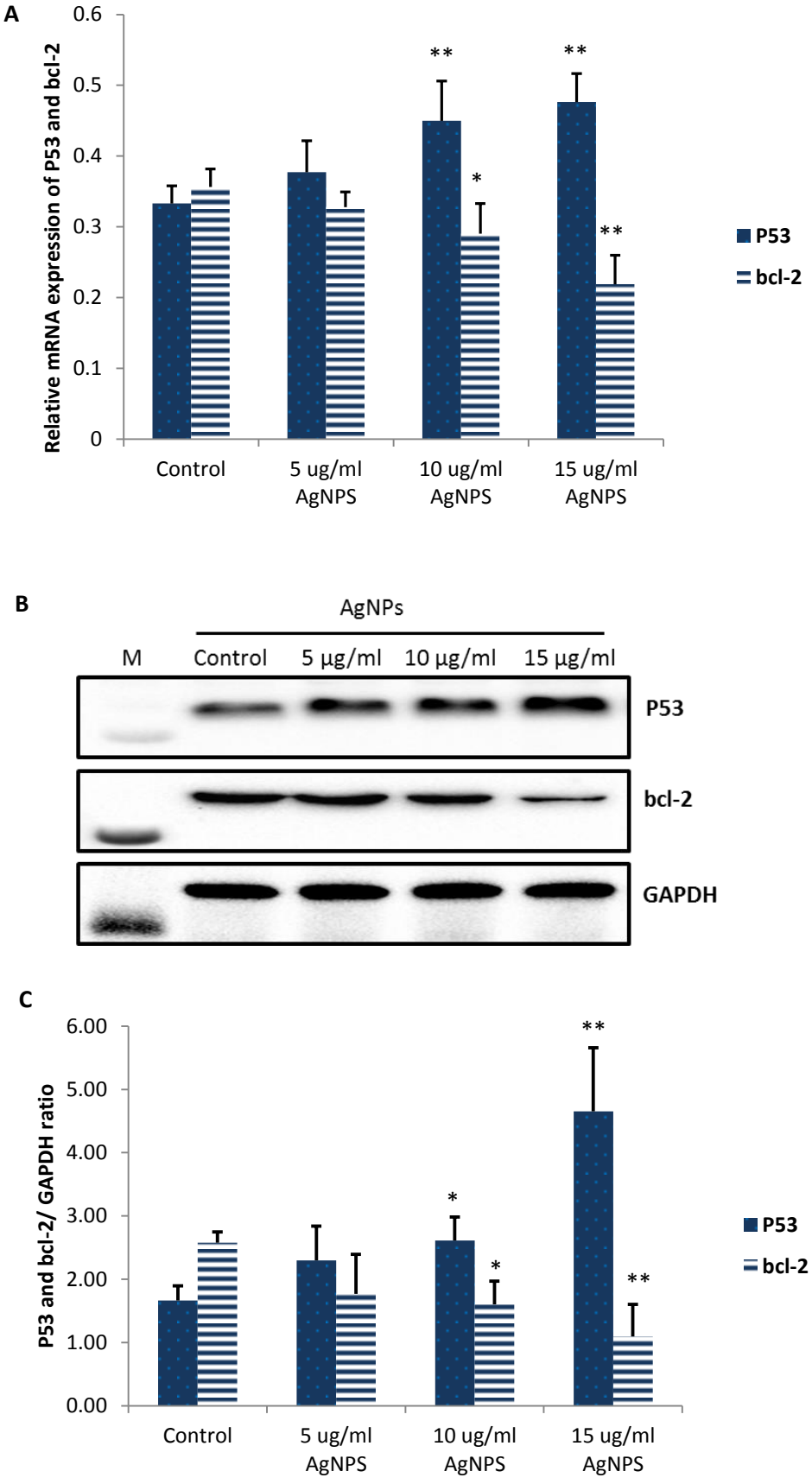

A
Relative mRNA expression of P53 and bcl-2
0.6
0.5
0.4
0.3
0.2
0.1
0
**
**
*
**
Control
5 ug/ml AgNPS
10 ug/ml AgNPS
15 ug/ml AgNPS
P53
bcl-2
B
AgNPs
M
Control
5 µg/ml
10 µg/ml
15 µg/ml
P53
bcl-2
GAPDH
C
P53 and bcl-2/ GAPDH ratio
6.00
5.00
4.00
3.00
2.00
1.00
0.00
**
*
*
**
Control
5 ug/ml AgNPS
10 ug/ml AgNPS
15 ug/ml AgNPS
P53
bcl-2

Figure 7

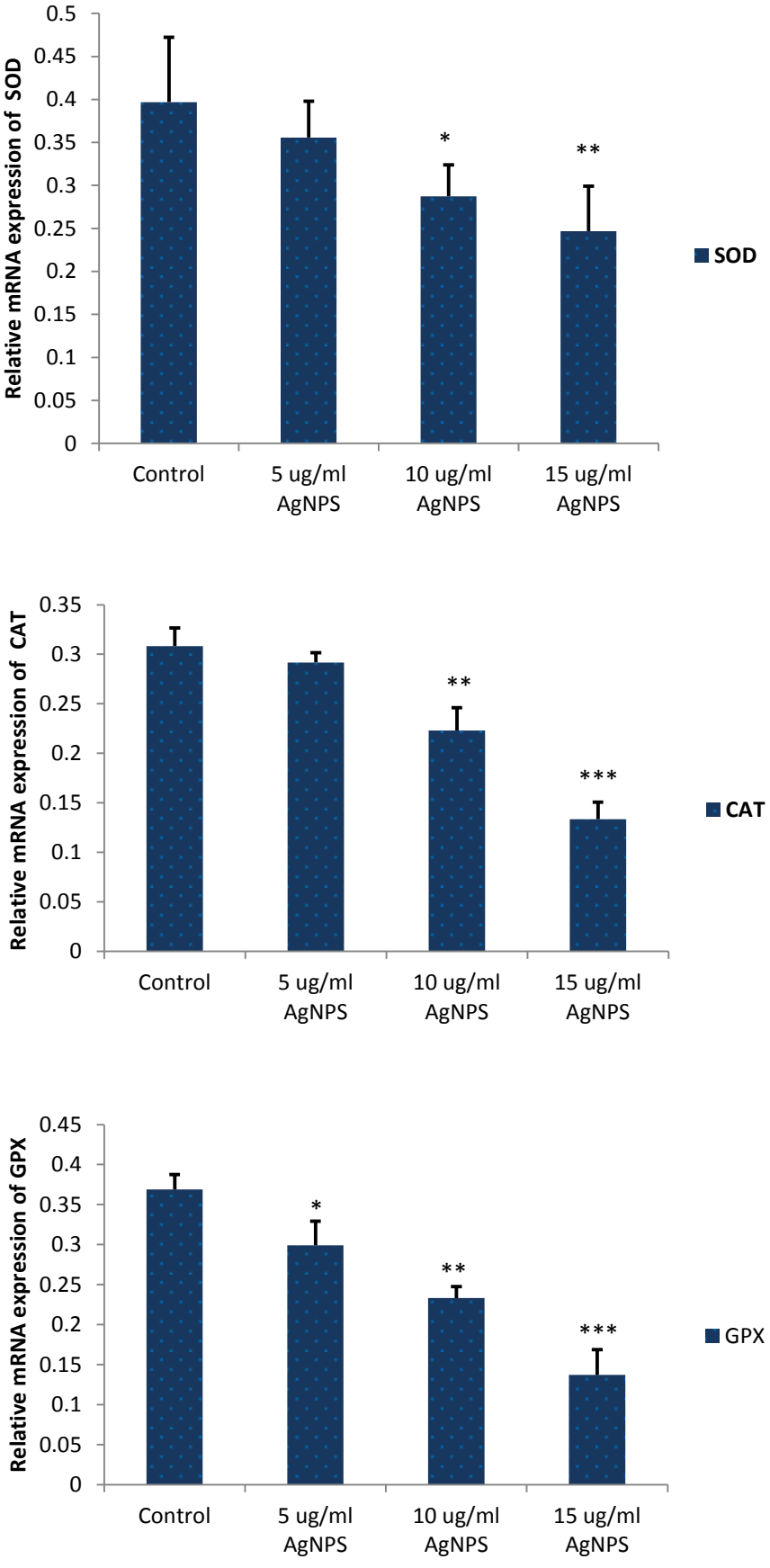
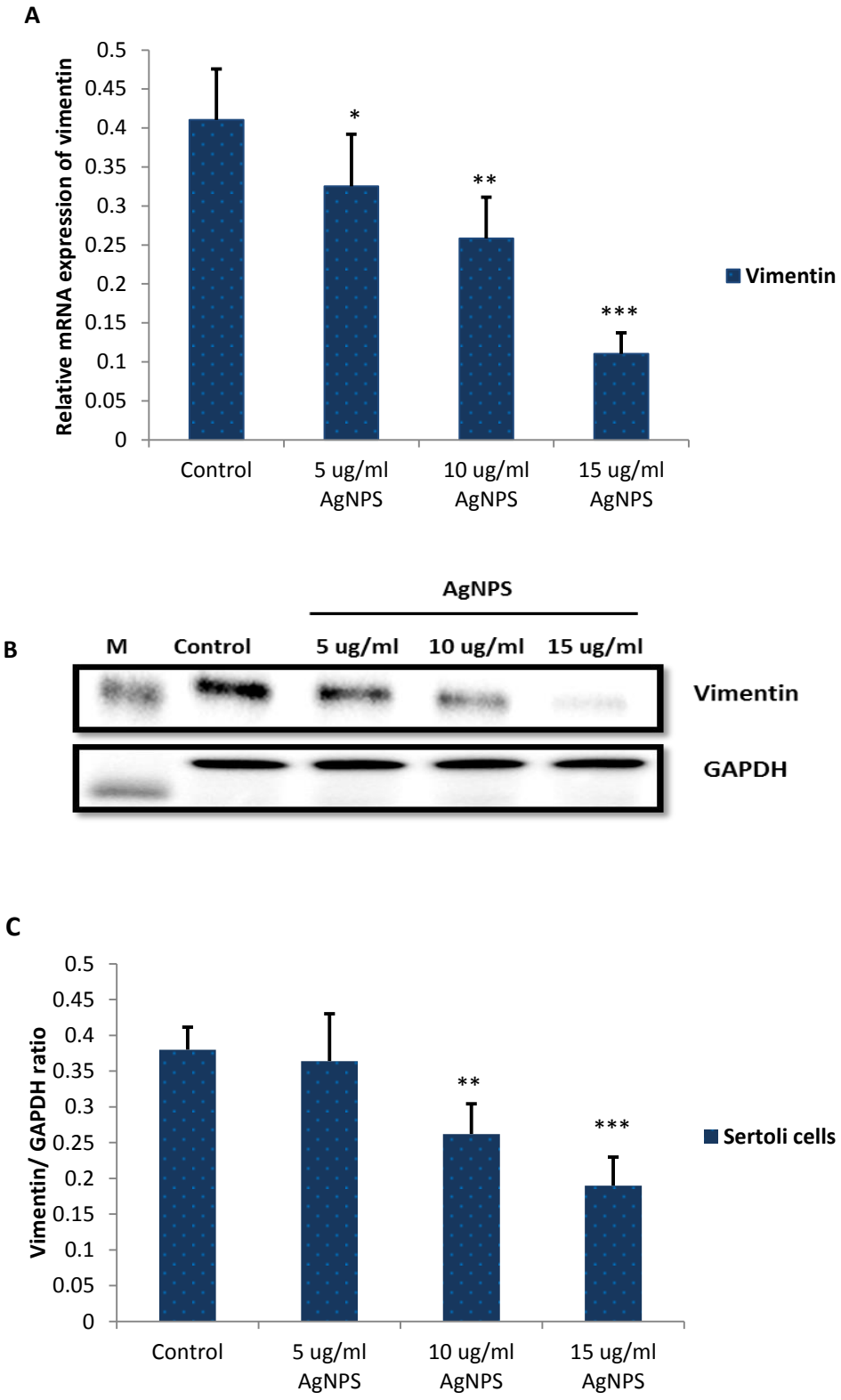

Relative mRNA expression of SOD
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
**
Control
5 ug/ml AgNPS
10 ug/ml AgNPS
15 ug/ml AgNPS
SOD
Relative mRNA expression of CAT
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
**
***
Control
5 ug/ml AgNPS
10 ug/ml AgNPS
15 ug/ml AgNPS
CAT
Relative mRNA expression of GPX
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
**
***
Control
5 ug/ml AgNPS
10 ug/ml AgNPS
15 ug/ml AgNPS
GPX

Figure 8


A
Relative mRNA expression of vimentin
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
**
***
Control
5 ug/ml AgNPS
10 ug/ml AgNPS
15 ug/ml AgNPS
Vimentin
B
AgNPS
M
Control
5 ug/ml
10 ug/ml
15 ug/ml
Vimentin
GAPDH
C
Vimentin/ GAPDH ratio
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
**
***
Control
5 ug/ml AgNPS
10 ug/ml AgNPS
15 ug/ml AgNPS
Sertoli cells